1 **Title:** Milk of cow and goat, immunized by recombinant protein
2 vaccine ZF-UZ-VAC2001(Zifivax), contains neutralizing antibodies
3 against SARS-CoV-2 and remains active after standard milk
4 pasteurization.

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24 Abstract

25 Here we report the first experimental validation of the possibility for obtaining immune
 26 milk with neutralizing antibodies against SARS-CoV-2 from vaccinated cow and goat
 27 using recombinant protein human vaccine, ZF-UZ-VAC2001. In the period of two weeks
 28 after first vaccination, we detected the neutralizing antibodies against coronavirus in the
 29 blood serum of vaccinated animals. The neutralizing activity, in its peak on the 21st
 30 days after receiving the third dose (77th day from first dose), was effective in
 31 Neutralization Test using a live SARS-CoV-2 in Vero E6 cells, even after 120-fold serum
 32 titration. Colostrum of the first day after 3rd dose vaccinated cow after calving had a
 33 greater activity to neutralize the SARS-CoV-2 compared to colostrum of subsequent
 34 three days (4.080 µg/ml vs 2.106, 1.960 and 1.126 µg/ml), goat milk (1,486 µg/ml), and
 35 cow milk (0.222 µg/ml) in MAGLUMI® SARS-CoV-2 neutralizing antibody competitive
 36 chemiluminescence immunoassay. We observed a positive correlation of receptor-
 37 binding domain (RBD)-specific IgG antibodies between the serum of actively immunized
 38 cow and milk-feeding calf during the entire course of vaccination ($r = 0.95$, $p = 0.027$).
 39 We showed an optimal regime for immune milk pasteurization at 62.5°C for 30 min,
 40 which retained specific neutralizing activity to SARS-CoV-2, potentially useful for
 41 passive immunization against coronavirus infection threats.

42 **Key words:** bovine immunoglobulins, SARS-CoV-2, immune milk, neutralizing
 43 antibody, passive immunization, vaccination, ZF-UZ-VAC2001.

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49 Dear Editor,

50 In the global coronavirus disease pandemic, immunological studies proved that
 51 antibodies are the effective molecules for sanitizing the body from viruses. However, the
 52 formation of novel SARS-CoV-2 mutations is leading to a decreased effectiveness of
 53 vaccines. Moreover, the slow rate of massive vaccination process, due to poor public
 54 acceptance and/or insufficient vaccine supplies in some countries, are the one of the
 55 main factors for continuous reemergence of new virus variants of concern (VOC). The
 56 development and registration of new vaccines against constantly emerging mutations
 57 require additional time and funding. This underlies to explore new opportunities to
 58 establish a stable herd immunity, focusing on the development of highly effective
 59 neutralizing antibodies (nAbs) [1]. Apparently, nAbs against VOC can be quickly
 60 obtained by the vaccination of farm animals with the emergency use approved (EUA)
 61 human vaccines, covering the new mutations of importance. Here, we are to briefly
 62 communicate the first experimental validation report on obtaining immune milk with
 63 neutralizing antibodies (nAbs) against SARS-CoV-2 from cow and goat after vaccination
 64 with RBD-based recombinant protein subunit human vaccine, ZF-UZ-VAC2001 or
 65 (ZF2001 or Zifivax).

66 The idea, to study and validate if vaccinated farm animal milk contains nAbs or not,
 67 came from the evidence that antibodies to SARS-CoV-2 were found in the milk of
 68 women who have had COVID-19 or been vaccinated [2]. We also carefully read and
 69 acknowledge several publications on the possible benefit from the passive immunization
 70 using milk of vaccinated cow. Jawhara [3] first suggested that microfiltered raw immune
 71 milk or colostrum collected from SARS-CoV-2 vaccinated cows could provide short-term
 72 protection against SARS-CoV-2 infection in humans. Further, Arenas et al. [4] proposed
 73 to use of heterologous passive immunity using Bovine Coronavirus (BCoV) immune milk
 74 as an immunostimulant therapy to control SARS-CoV-2 infection because vaccination of

75 farm animals is a well-known and has been described in the literature to protect animals
76 from diseases, including BCoV [5]. Gallo et al. [6] reviewed the antiviral properties of
77 native and chemically modified whey proteins and their potential applications in human
78 health, focusing on their application in prevention and treatment of SARS-CoV-2
79 infection.

80 However, neither the detection of nAbs in the serum and milk of vaccinated household
81 farm animals using human vaccines approved for emergency use, nor the effect of
82 pasteurization of such immune milk on the SARS-CoV-2 virus-neutralizing activity, has
83 not yet been experimentally validated. To address these limitations, we first performed a
84 series of pilot experiments in 60 milk and serum samples of vaccinated household cows
85 and goat. In that, healthy pregnant as well as dairy cow with feeding calf were
86 vaccinated with the ZF-UZ-VAC2001 recombinant SARS-CoV-2 human vaccine,
87 containing a dimeric form of the receptor-binding domain (RBD) as the antigen [7].

88 We used this particular vaccine because it was successfully passed a 3rd phase clinical
89 trial in Uzbekistan and were approved for the massive vaccination of the population [8].
90 ZF-UZ-VAC2001 has shown its effectiveness against the diverged variants of the SARS
91 CoV-2 virus [8,9], namely Alfa (92.93%), Gamma (100%), Delta (77.47%), and Kappa
92 (90.0%). Although the neutralization activity of currently used human vaccines, has
93 shown a decreased effect against emerging new VOC B.1.1.529 (Omicron) variant [9],
94 the prolonged time between the second and third dose injections of ZF-UZ-VAC2001
95 (0,1, 2 vs 0, 1, 5 months) has revealed a better neutralization effect against Omicron
96 variant [10].

97 In our study, we performed vaccination of farm animals (cows and goat) with
98 revaccination carried after 28 and 56 days. Examination of the udder, palpation of the
99 lymph nodes, mucous membranes and measurement of rectal temperature as well as

100 the observation of the behavior of animals have not revealed any side effects during
101 daily monitoring of animals after vaccination.

102 Since the first dose vaccine injection period, we have regularly collected and evaluated
103 the virus neutralization activity of the immune milk and blood serum of the cow and
104 goat, measuring the inhibition level of viral RBD binding to the ACE2 receptor. For this
105 purpose, we used the MAGLUMI® SARS-CoV-2 Neutralizing Antibody competitive
106 chemiluminescence immunoassay (CLIA). Milk and blood serum from the non-
107 vaccinated cows were used as control samples. For blood serum samples, we
108 performed the direct neutralization test using a live SARS-CoV-2 proliferating in Vero E6
109 cells [S1] at the Medical University of Vienna, Austria. We also determined antigen-
110 specific antibody isotypes to the RBD of spike (S) and full-length S proteins in bovine
111 serum and milk samples using enzyme-linked immunosorbent assay (ELISA) analysis
112 (see Supplementary Materials and Methods).

113 We detected the neutralizing antibodies to the RBD domain and S-protein of SARS-
114 CoV-2 in the blood serum of a vaccinated animal as early as two weeks after the first
115 vaccination. Results showed that revaccination contributed to an increase in the effect
116 of inhibition. We noted maximum neutralization activity of blood serum (100%) and milk
117 (40%) on the 77th day from the date of the first vaccination. The correlation between
118 neutralization rate of cow sera and milk during the entire course of vaccination was
119 significant at $r = 0.96$, $p = 0.022$

120 Complete viral neutralization of the cytopathic effect on Vero E6 cells was detected
121 even with 120-fold serum titration. The blood serum and milk of the vaccinated cow
122 contained specific IgG to the RBD domain and S-protein of SARS-CoV-2.

123 We also found statistically significant correlation between the level of IgG specific to the
124 RBD domain and the neutralization rate of milk of vaccinated cows ($r = 1.0$, $p = 0.001$,
125 Supplementary Table 1).

126 When we vaccinated other group of “Simmental” cows during the dry period of the third
127 trimester of pregnancy, after calving we determined that that the colostrum of the first
128 day had a greater ability to neutralize the SARS-CoV-2 virus (4.080 µg/ml) compared to
129 colostrum of subsequent next days (2.106, 1.960 and 1.126 µg/ml) and milk [0.222
130 µg/ml that was within the range of 50% (or 0.30 µg/ml) neutralization activity].

131 In another pilot experiment in a domestic lactating goat (*Capra hircus*), it was sufficient
132 to use only 2 dose injection of the vaccine to achieve the effect of immunization and
133 obtain immune milk samples. The correlation between neutralization rate of goat sera
134 and goat milk in during the entire course of vaccination was high at $r = 0.99$, $p = 0.003$
135 (Supplementary Table 1). The maximum of virus-neutralizing activity of the immune
136 goat milk was 1.486 µg/ml on the 14 days of the third dose vaccine injection.

137 We further investigated the possibility of transferring of SARS-CoV-2 specific IgG
138 antibodies via milk from cow to its milking calf. Toward this goal, we performed the
139 analysis of sera from calf during active vaccination of cow. Results showed that there is
140 statistically significant correlation ($r = 0.95$, $p = 0.027$, Supplementary Table 1) between
141 the level of IgG specific to RBD of vaccinated cow and calf fed by milk of vaccinated
142 mother cow. This showed the possibility of passive immunization using milk of
143 vaccinated mother cows, containing specific IgG against SARS-CoV-2.

144 In perspectives, as highlighted above [2-6], there is a huge opportunity to use the
145 passive immunization property of immunized cow milk in humans. For this, there is a
146 need for determining the optimal pasteurization conditions of immune milk to keep its
147 biologically active neutralization against SARS-CoV-2. Toward this goal, we have
148 studied several pasteurization regimes of immunized milk while analyzing its
149 neutralization activity of SARS-CoV-2. We tested 20 milk samples obtained from
150 vaccinated cow and goat, pasteurizing them at different temperatures and regimes of

151 pasteurization according to State regulations (62.5-63°C for 30 min, 72°C for 5 min,
152 85°C for 5 sec).

153 As expected, temperature treatment for pasteurization of milk product negatively
154 correlated with neutralization activity ($r = -1.0$, $p = 0.02$). Compared to before-
155 pasteurization raw milk (0.5 ± 0.2 µg/ml), pasteurization treatments at 72 °C (for 5 min)
156 and 85 °C (for 5 sec) temperatures showed sharp activity decreases (0.1 ± 0.04 µg/ml
157 and 0.05 ± 0.01 µg/ml respectively). However, the pasteurization at 62.5-63 °C during 30
158 minutes retained sufficiently active neutralization property in nAbs chemiluminescence
159 immunoassay (0.3 ± 0.1 µg/ml), small decrease observed between raw and pasteurized
160 immune milk at 62.5-63 °C during 30 minutes was statistically non-significant ($p = 0.24$;
161 (Fig.1; Supplementary Table 2).

162 Thus, these first experimental validation results indicated that the ZF-UZ-VAC2001
163 human vaccine can be safely used for the vaccination of household farm animals to
164 obtain immuno-biologically active milk and serum, containing specific nAbs against
165 SARS-CoV-2. There is no need for the substantial corrections in dose calculations for
166 vaccination of the animals, which can be performed in accordance to the manufacturer's
167 instructions and protocols for human vaccination. Interestingly, we experimentally
168 observed that milk and serum of vaccinated cow not only have active neutralization
169 property against SARS-CoV-2, but also biologically active specific IgG antibodies to
170 RBD and S protein, transferred from mother cow milk to its milking calf, passively
171 forming offspring's immune protection.

172 We found that the most optimal condition for immunized milk pasteurization can be
173 achieved 62.5-63 °C during 30 minutes, which retains efficient virus neutralizing
174 antibody activity against SARS-CoV, providing future opportunity for the passive
175 immunization of human by milk consumption. The limitation of this study is a small
176 number of farm animals included for vaccination experiments and future large-scale

177 experiments are required, which is in progress in our laboratories. However, early
178 results collectively showed that pasteurized immune milk obtained from vaccinated
179 household animals using human vaccine against SARS-CoV-2, administered herein,
180 potentially could be useful for passive immunization against coronavirus infection
181 threats. The use of immune colostrum, milk and dairy products with neutralizing
182 antibodies from vaccinated cows and goats seems to be a promising approach for the
183 preparation of safe and natural prophylactic agents against human and animal
184 infections.

185

186 **Abbreviations**

187 ACE2: angiotensin-converting enzyme 2; COVID-19: coronavirus disease; ELISA:
188 enzyme-linked immunosorbent assay; HRP: horseradish peroxidase; IgG, IgA, IgM:
189 immunoglobulin G, A, M; nAbs: neutralizing antibodies; NT: Neutralization test; OD:
190 optical density; RBD: receptor-binding domain; S: spike protein; S1: spike protein
191 receptor-binding subunit; SARS: severe acute respiratory syndrome; VOC: variants of
192 concern; CLIA: chemiluminescence immunoassay; EUA: emergency use approval; ZF-
193 UZ-VAC2001: Zhifei (ZF) – Uzbekistan (UZ) –Vaccine (VAC) project started in January
194 of 2020 (20/01).

195 **Supplementary Information:**

196 The online version contains supplementary material available at <https://doi.org/xxx>

197 **Supplementary Table 1.** Statistical analysis of correlations between parameters.

198 **Supplementary Table 2.** P-values of significant difference between nAbs levels of
199 immune milk at different pasteurization (n=20).

200 **Supplementary Materials and Methods.**

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212 **Authors' contributions**

213 **VG** and **IYA**: project administration, conceptualization, methodology, investigation, data
 214 curation, and writing—original draft, review and editing; **FYG**: consultation,
 215 methodology, writing—review and editing; **YL** and **ST**: methodology, investigation,
 216 writing—review and editing; **SK GB, KK, MR, NM, and AB**: investigation, data collection
 217 and curation. All authors contributed to the article and approved the submitted version.

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224 **Availability of data and materials**

225 The datasets used and/or analyzed during the current study are available from the
 226 corresponding author on a reasonable request.

227 **Declarations:**

228 **Ethics approval and consent to participate**

229 The studies reviewed and approved by the Ethics Committee Republic of Uzbekistan for
230 SARS-CoV-2 research (authorization number 6/6 1449 from 13.10.2020)

231 **Consent for publication**

232 All authors consent to the publication of the manuscript in Nutrition Journal.

233 **Competing interests**

234 The authors declare that the study concept and results are filed for patenting at the
235 Intellectual Property Agency under the Ministry of Justice of the Republic of Uzbekistan
236 with pending applications № IAP 2021 0365 9 and IAP 2022 0054.

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273 **Fig. 1 The box plots of SARS-CoV-2 Neutralizing Antibody levels ($\mu\text{g/mL}$).** Average
 274 indices (y-axis) for before pasteurization (raw) and pasteurized immune milk samples at
 275 the different regimes of pasteurization (x-axis) are shown. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq$
 276 0.01; ns: not statistically significant difference. Negative control represents milk sample
 277 from non-vaccinated animal. The box plots were generated, using GraphPad Prism 8
 278 (GraphPad Software Inc.).

SARS-CoV-2 Neutralizing Antibody, $\mu\text{g/mL}$